EFFECTS OF PINEALECTOMY ON HYPOTHALAMIC METABOLIC AND CLOCK GENE RHYTHMS

A Senior Scholars Thesis

by

Amanda Clauson

Submitted to the Office of Undergraduate Research
Texas A&M University
In partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

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Major: Cell and Molecular Biology

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Associate Dean for Undergraduate Research: Robert C. Webb

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ABSTRACT

Effects of Pinealectomy on Metabolic and Hypothalamic Clock Gene Rhythms

(April 2006)

Amanda Clauson
Department of Biology
Texas A&M University

Research Advisor: Dr. Vincent Cassone
Department of Biology

A neuroendocrine loop model has been proposed for explaining the generation of avian circadian rhythms. The basis of this model is that the circadian system is composed of interconnected circadian pacemakers residing within the suprachiasmatic nucleus (SCN) complex, retina and pineal gland, which control the phase of rhythmicity of peripheral oscillators in the absence of photic or neuroendocrine input from the rest of the system (Cassone and Menaker, 1984). However, recent data question the validity of the neuroendocrine loop model by suggesting that although melatonin administration affects overt clock function, (Lu and Cassone, 1993) it has no effect on the transcription of clock genes (Yasuo et al., 2002).

Thus, expression of these genes could be independent of pineal function. My research attempts to elucidate whether metabolic rhythms in the vSCN and clock gene expression in the vSCN and mSCN are coupled and to reveal the effects of pineal melatonin on these rhythms. This is done by comparing the affects of pinealectomy on 2DG uptake and canonical "clock" gene expression in the hypothalamus of house sparrows, *Passer domesticus*. Since other brain structures exhibit metabolic and clock gene rhythms, investigation of clock gene expression and 2DG uptake will also be undertaken in peripheral structures, e.g. the habenula and optic tectum. Currently, analysis of 2DG uptake at Day 0 has been completed and the process of analysis for Day 10 has begun. Using *in situ* hybridization techniques, I will quantify clock gene expression in both the vSCN and mSCN to determine if clock gene rhythms persist despite the absence of the pineal gland, the source of rhythmic melatonin for passerine birds. Because *in situ* analysis must be done from all timepoints and conditions simultaneously, and given the breadth of this experiment, these analyses are still being undertaken.

DEDICATION

This thesis is dedicated to my parents Dale and Sherry Clauson, who have taught me that success is only rewarded through hard work and determination.

NOMENCLATURE

Biological clock: An endogenous timing mechanism that controls the rhythmicity of physiological and behavioral functions.

Circadian rhythm: A biological rhythm with an approximately 24-hour period that continues in constant conditions.

Entrainment: The process by which an environmental cycle sets the period and phase of a self-sustained oscillator each day.

Free-running rhythm: A rhythm that persists in the absence of an environmental cycle.

Hypothalamus: The part of the brain that lies below the thalamus, forming the main portion of the ventral region of the diencephalon and functioning to regulate body temperature, metabolic processes and other autonomous activities.

Melatonin: An indolamine hormone synthesized and secreted by the pineal gland and retina in a precise circadian pattern.

Oscillator: A system of components that interact to produce a rhythm with a definable period length. Oscillators require a pacemaker for its entrainment and function.

Pacemaker: An oscillator that drives the output or entrainment of another oscillator. A circadian pacemaker is a specialized oscillator that operates quasi-independently of other oscillators, either directly or through other oscillators, and is entrained by environmental cues.

Phase: The instantaneous state of an oscillation relative to a reference point.

Pineal Gland: A small endocrine gland that forms as a diencephalic envagination and secretes melatonin. It is located between the two hemispheres of the brain, near the center.

Suprachiasmatic nucleus: A region of the brain that sits directly on top of the optic chiasm in the anteroventral region of the hypothalamus. It is necessary for the generation of endogenous circadian rhythms.

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1. INTRODUCTION[1]

Biological rhythms and the clocks that control them are fundamental to the physiology and behavior of most organisms. In vertebrates, control of biological rhythmicity is regulated by several neural and neuroendocrine structures (Gaston and Menaker, 1968). Through a series of experiments, the avian pineal gland was found to be a circadian pacemaker, which, via its secretion of melatonin, regulates the period and phase of overt circadian rhythms and imposes circadian temporal order on a set of subsystems within the house sparrow, *Passer domesticus* (Zimmerman et. al., 1979, Ebihara et al., 1984; Cassone et al., 1992; Lu and Cassone, 1993). In spite of its clear role as a pacemaker, there is extensive evidence that the pineal gland is only part of a complex system of structures controlling avian circadian rhythms. In mammals, the major pacemaker controlling circadian rhythms is located in the hypothalamic suprachiasmatic nucleus (SCN) (Klein, 1991). In birds, two structures have been touted as avian homologues to the SCN. First is a structure in the medial hypothalamus that has been designated the medial suprachiasmatic nucleus (mSCN), (Brandstatter and

[1] This thesis follows the style and format of Journal of Neuroscience.

Abraham, 2003) and which expresses clock genes rhythmically, but does not exhibit any known physiological rhythmicity (Yasuo et al., 2002; Abraham et al., 2003). Second is a structure located lateral to the mSCN that has been designated the visual suprachiasmatic nucleus (vSCN) by some (Cassone and Moore, 1987), and expresses circadian rhythms in 2DG uptake (Cantwell and Cassone, 2002). It also has been found to have a high density of melatonin receptors (Rivkees et al., 1989) and clock gene expression, as reported in house sparrows (Abraham et al., 2002).

Based on several studies focusing on the roles of these structures in circadian organization, the Cassone laboratory proposed a "Neuroendocrine loop model" governing the regulation of the complex avian circadian system (Figure 1). The premise of this model is that the circadian system is composed of interconnected dampened circadian oscillators residing within the SCN, pineal gland and retina which are capable of self-sustained oscillation in the presence of photic or neural endocrine input from the rest of the system. These pacemakers rely on their mutual interactions to maintain stability in the phases and amplitude of the overt circadian rhythm. In sparrows, the SCN is metabolically active during the subjective day and inhibits melatonin synthesis in the pineal gland, allowing the synthesis and secretion of

melatonin only at night. Melatonin secreted into the bloodstream and cerebral spinal fliud inhibits metabolic activity within the SCN through the action of several melatonin receptors.

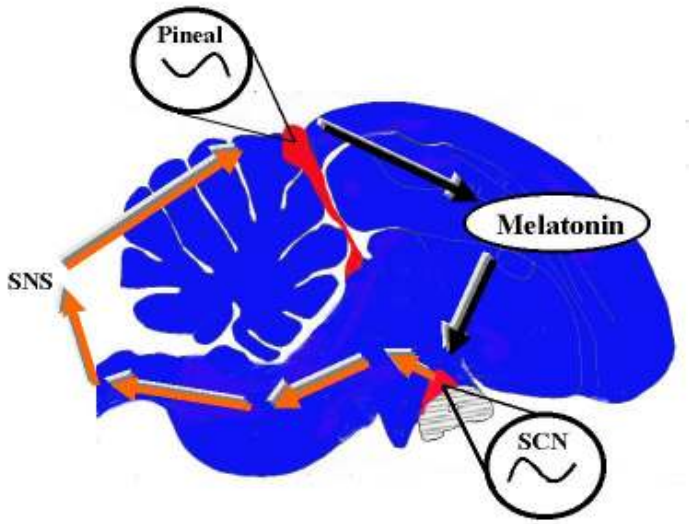

Figure 1: Neuroendocrine Loop Model

Shown above is a neuroendocrine loop model of avian pacemaker organization in a generalized avian brain. This representation shows the pineal gland and suprachiasmatic nucleus (consisting of the vSCN and mSCN), each of which are dampened pacemakers that mutually interact to maintain circadian rhythmicity.

However, recent data question the validity of the neuroendocrine loop model by suggesting that although melatonin administration affects overt clock function, (Cassone and Lu, 1992) it has no apparent effect on the transcription of clock genes

(Yasuo et al., 2002). Thus, the present experiment seeks to test the effects of pineal gland removal, which in essence removes the source of endogenous melatonin, on metabolic rhythms and rhythms of clock gene expression in an effort to determine if these rhythms are independent from each other. The first clock gene to be investigated is *PdClock,* a positive component of the neuroendocrine loop that is expressed rhythmically and peaks late in the subjective day. *PdPer2* is the second clock gene that will be investigated*,* a negative component of the loop that is also expressed rhythmically in the pineal gland and SCN.

2. MATERIALS AND METHODS

Pinealectomies

House sparrows were collected and communally housed in an indoor aviary. Sparrows were placed in cages inside light-tight environmental chambers with either infrared motion detectors or perches connected to micro switches to monitor locomotor activity. The sparrows were kept under a 12:12 light:dark cycle (LD). These detectors transmit activity data to the QA4 activity modules of the Minimitter Dataport 24 interface. Following one week of acclimatization to the cages, 80 birds were anesthetized with a ketamine/xylazine combination, and half were surgically pinealectomized (PINX). Pinealectomy consists of removal of the feathers and incising the skin. A small hole is drilled into the top of the skull and the skullcap is temporarily removed. The meninges are retracted, and the pineal gland is removed with forceps. The skullcap is replaced and the skin sutured back together. The other half of the birds received a sham surgery (SHAM), which is the same procedure without removing the pineal gland. Following one week of recovery, the birds were placed in constant darkness (DD). On Day 0 of DD all birds are rhythmic (Gaston and Menaker, 1968;

Cassone and Lu, 1992). At CT 5, five PINX and five SHAM birds received intramuscular injections of 200 μCi/kg ^{14}C-2-deoxy-glucose (2DG) and at CT 6, the midpoint of the activity phase, were sacrificed in the dark by CO_2 asphyxiation. This procedure was repeated at Day 10 of DD, where SHAM birds were rhythmic but the PINX birds were arrhythmic. The time of CT 6 was projected using a triangulation of the actogram data. This procedure will also be repeated at Day 3 and Day 7 of DD where five SHAM and five PINX birds will be injected with 2DG, sacrificed, and brains removed. The entire procedure is repeated at CT 17 (with the remaining forty birds).

Tissue Processing

Brains were removed rapidly and frozen in -40°C isopentane. Brains were sectioned through the pre-optic and hypothalamic regions on a cryostat at -20° C at 25 μm. Four bins were cut to ensure that adjacent sections can be analyzed for 2DG autoradiography, *Pdclock* and *Pdper2 in situ* hybridization, and a control. The first bin was thaw mounted to charged slides and apposed to radiographic film for seven days. The images were then digitally captured, densitized and quantified relative to a known amount of ^{14}C (Cassone et. al., 1988).

In-situ Hybridizations

The second and third bins will be probed with the cRNA probes. The Cassone laboratory has created anti-sense riboprobes against *Pdper2* and *Pdclock*. These two genes represent one element from each of the positive and negative arms of the molecular circadian loop. Controls will be performed on the fourth bin using the sense strands of the respective riboprobes. All of the autoradiographs will be viewed on a Phosphor imager and then committed to film. Both the *in situ* and 2DG autoradiographs are to be densitized and quantified relative to a known amount of ^{14}C (Cassone et. al., 1988).

3. RESULTS

Day 0 sparrows that were pinealectomized or received the sham surgery were found to be rhythmic at the time of tissue extraction. Day 10 SHAM sparrows, after placement into constant darkness, were found to have clear free-running rhythms for the entire ten days. Pinealectomized sparrows, after switching from an 12:12 LD cycle to constant darkness, exhibited free running rhythms for approximately five days and then became arrhythmic (Figure 2).

Eight structures in the brain were analyzed for 2DG uptake (Figure 3). The first two, the mSCN and vSCN, are the circadian pacemakers that comprise one of the components of the neuroendocrine loop. The other six structures are peripheral structures in the brain that also display rhythmicity in metabolic activity, including the nucleus geniculatus lateralis, pars ventralis (GLv), ectostriatum (E), nucleus rotundus (ROT), nucleus pretectalis (PT), optic tectum (TeO), and nucleus habenularis lateralis and medialis (HL and HM).

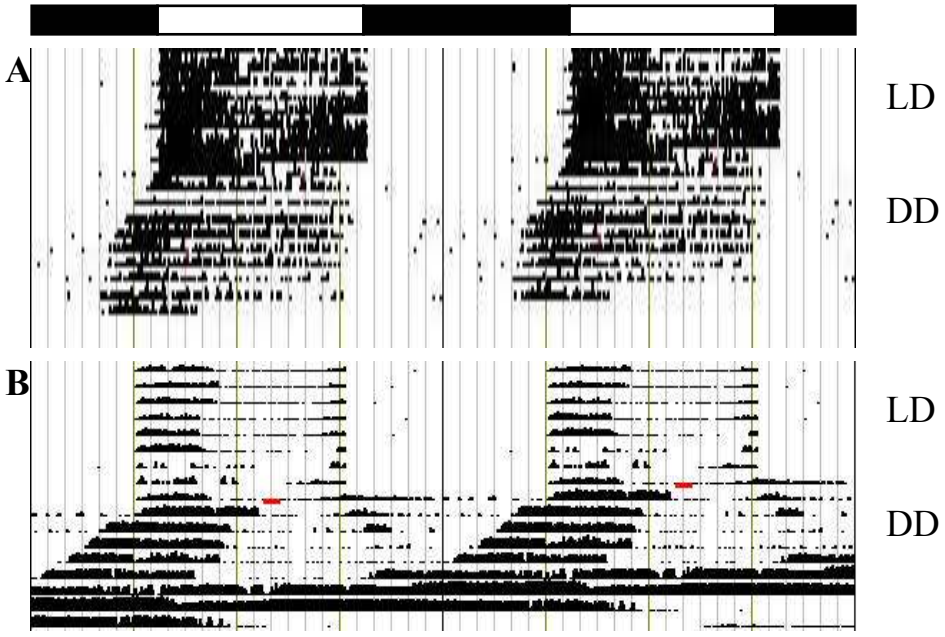

Figure 2: Locomotor Activity of House Sparrows

The top actogram (A) shows the activity of a SHAM bird. When placed in DD, the bird exhibits clear free running rhythms. Actogram B is the locomotor record of a PINX bird. When switched from an LD light cycle to constant darkness, the sparrow's activity becomes free running for a few days and then becomes arrhythmic.

The Day 0 CT6 pinealectomized sparrows were found to have a higher uptake of 2DG than the CT6 sham surgery sparrows in all of the brain structures analyzed (Figure 4). In the vSCN, the trend shows that there is greater uptake of 2DG in the PINX birds, with an average density measurement of 0.80 μCi/g compared to an average of 0.44 μCi/g for SHAM birds (N=5). In the mSCN the same trend was found, with the average density measurement in PINX birds being 0.74 μCi/g and in SHAM

birds 0.36 μCi/g. In the HL and HM, there was found to be a significant difference between the CT6 PINX and control birds, with the average ^{14}C uptake being 0.92 μCi/g and 0.38 μCi/g, respectively ($p=0.002$). Similar trends were found in the remaining brain structures.

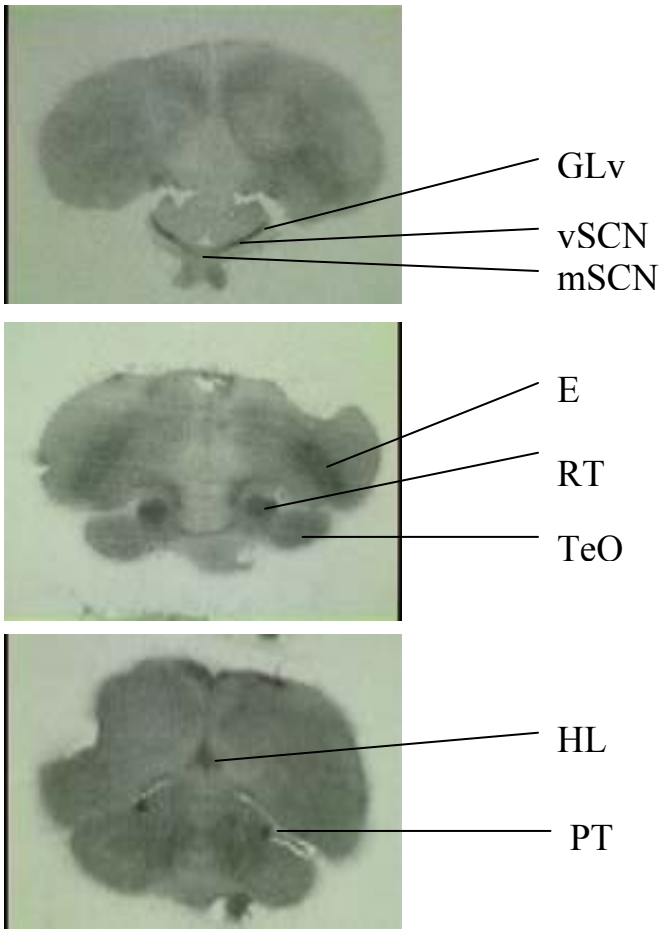

Figure 3: Morphology of the Brain

Autoradiographs illustrating the neuroanatomy of the pre-optic and hypothalamic regions of the brain.

An identical trend was found with the CT18 sparrows when comparing metabolic activity between the SHAM and PINX birds. In the vSCN the average density measurement for the PINX birds was 0.73 μCi/g and SHAM birds was 0.51 μCi/g ($p=0.045$). In the mSCN, a significant difference was also found between PINX and SHAM birds with density measurements of 0.56 μCi/g and 0.39 μCi/g, respectively ($p=0.042$). In the optic tectum, a significant difference was found, with the average uptake for PINX birds being 0.67 μCi/g and for SHAM birds 0.37 μCi/g ($p=0.032$). Also in the ROT, the average uptake for PINX sparrows was 0.82 μCi/g, while in SHAMS it was 0.48 μCi/g ($p=0.016$). Similar trends were found with the remaining brain structures.

When comparing metabolic activity between the CT6 and CT18 pinealectomized birds, CT6 sparrows exhibited a higher 2DG uptake in all of the brain structures where density measurements were taken (Figure 5). This trend was shown in the vSCN, where the average uptake in PINX versus SHAM sparrows was 0.80 μCi/g and 0.73 μCi/g, respectively. In the mSCN the same relationship was found, with an average density of ^{14}C measured as 0.74 μCi/g in PINX birds and 0.56 μCi/g in the control birds.

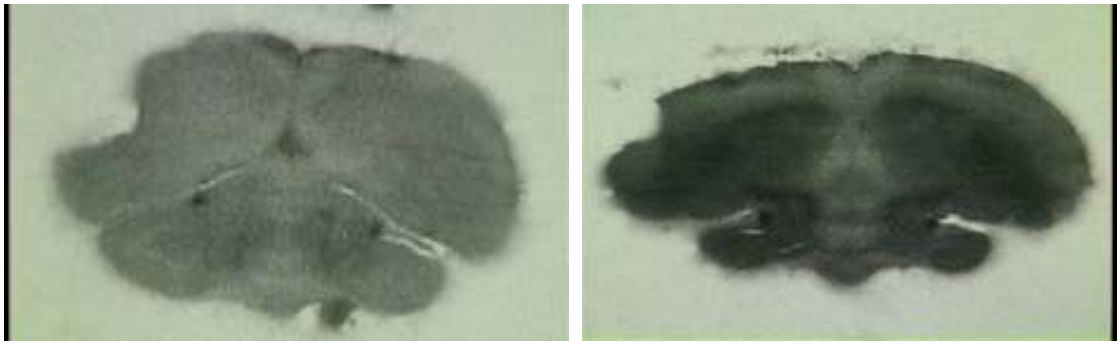

Figure 4: PINX versus SHAM sparrow

Shown are the autoradiographs of a SHAM sparrow at CT 6 (left) and a PINX sparrow at CT 6 (right). When the pineal gland is removed, the uptake of 2DG in the brain increases relative to the sham surgeries.

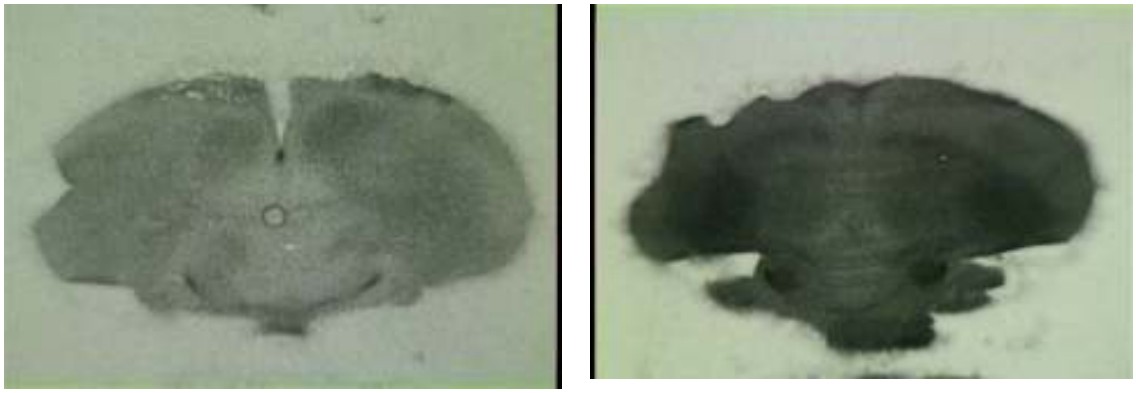

Figure 5: CT 18 versus CT 6 sparrow

Shown are the autoradiographs of a CT 18 PINX (left) versus CT 6 PINX (right) sparrow. The sparrow sacrificed at the midpoint of the activity phase has higher metabolic activity than the sparrow sacrificed during the night.

A similar relationship was found with CT6 and CT18 sham birds; higher metabolic activity was found for the birds sacrificed during the midpoint of the activity phase than the birds where the brain was extracted during the midpoint of the subjective night. In the vSCN, this correlation was found again with the average uptake in for shams at CT 6 0.51 μCi/g and in shams at CT 18 0.44 μCi/g. In the Glv, a strong trend showed this relationship, with CT6 sparrows having an average ^{14}C uptake of 0.63 μCi/g and CT18 sparrows 0.44 μCi/g. While not all of the differences were found to be significant, normalization of these data to the amount of 2DG in the blood of individual sparrows has not yet been performed; when completed, the results not yet shown to be statistically significant are expected to become so.

4. CONCLUSION

Conclusion

The pineal gland functions as a pacemaker for passerine birds. It was found that surgical removal of the pineal gland abolishes the free running rhythms in locomotor activity in house sparrows (Zimmerman and Menaker, 1979). However, the locomotor activity of pinealectomized birds can be entrained to LD cycles, and it was found that the SCN is necessary for the maintenance of circadian rhythmcity (Moore, 1978). Thus, the present experiment seeks to determine the relationship between these two components and determine if metabolic activity and clock gene expression in the SCN is driven by the pineal gland.

Pinealectomized sparrows exhibited higher 2DG uptake than sham sparrows. This is consistent with the neuroendocrine loop model and previous data from the Cassone lab (Lu and Cassone, 1992). The pineal gland secretes melatonin, which, among its wide array of effects, inhibits the metabolic activity of the SCN during the subjective night. In the absence of the pineal gland, no melatonin is secreted and thus metabolic activity in the SCN is disinhibited. Therefore, uptake of 2DG is greater in

these regions after pinealectomy. When comparing SHAM and PINX CT 18 sparrows, this is evident. Normally the SCN is inhibited at night, but with no melatonin being secreted, the SCN is disinhibited, and it in turn affects output via several pathways, and overall metabolic activity is increased.

CT 6 sparrows were also found to have higher metabolic uptake than CT 18 sparrows when comparing CT 6 PINX to CT 18 PINX or CT 6 SHAM to CT 18 SHAM. This is also consistent with the neuroendocrine loop model. The SCN is an oscillator that is metabolically active during the subjective day, but spontaneously wanes in activity until dusk, when it possesses very little activity. Therefore, sparrows sacrificed during the day should have a more metabolically active SCN region and higher 2DG uptake than CT 18 birds, which was found to be the case.

Future Studies

Due to time constraints, this experiment has not yet been completed. Day 10 sparrow data is in the process of being analyzed for 2DG uptake. Surgeries, tissue extraction and brain sectioning is still being completed for Day 3 and Day 7 sparrows. These must also be committed to film and densitized to a known amount of ^{14}C. After obtaining brain sections for Day 3, 7 and 10 for both SHAM and PINX birds taken at

both CT 6 and CT 18, *in situ* hybridizations will be performed (Figure 6). This will allow for comparison of metabolic rhythms to rhythms in clock gene expression and help explain how the pineal gland not only has a role in circadian rhythms but is also directly related to metabolic function. I plan on continuing this project in the fall of the coming year.

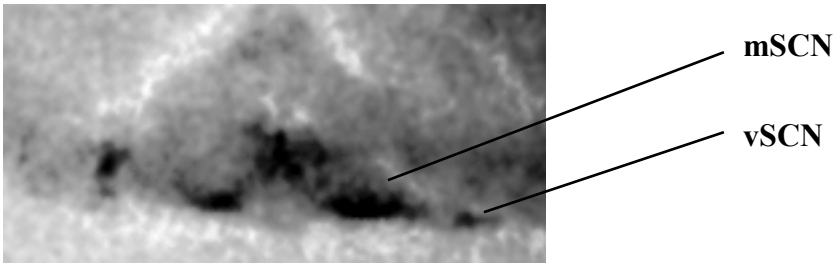

Figure 6: *In situ* Hybridization
Autoradiograph of the pre-optic hypothalamus showing the location of the *in situ* hybridization signal with *PdPer2* antisense probe.

I hypothesize that both 2DG uptake and clock gene expression will remain rhythmic in all SHAM birds. In pinealectomized birds, I expect 2DG uptake in the vSCN to have diminished by Day 3 and have no rhythm apparent by Day 10, based upon previous data (Cassone and Lu, 1992). I anticipate the clock gene expression to still be rhythmic after Day 3, as previously demonstrated (Abraham et al., 2003).

However, I also expect clock genes to be arrhythmic by Day 10, illustrating that although clock gene expression is less sensitive to PINX than metabolic activity is, PINX still eventually disrupts clock gene rhythms in addition to metabolic rhythms. I expect the rhythms in peripheral tissues to dampen within a few days, faster than would occur in the vSCN and mSCN where the circadian pacemakers are located, since these structures are directly sensitive to pineal melatonin.

REFERENCES

Abraham U, Albrecht U, Brandstatter R (2003) Hypothalamic circadian organization in birds. Chronobiol Int 20:657-69.

Abraham U, Albrecht U, Gwinner E, Brandstatter R (2002) Spatial and temporal variation of passer Per2 gene expression in two distinct cell groups of the suprachiasmatic hypothalamus in the house sparrow (Passer domesticus*).* Eur J Neurosci 16:429-36.

Brandstatter R, Abraham U (2003) Hypothalamic circadian organization in birds. I. Anatomy, functional morphology, and terminology of the suprachiasmatic region. Chronobiol In. 29:637-55.

Cantwell EL, Cassone VM (2002) Daily and circadian fluctuation in 2-deosy-glucose uptake in circadian and visual system structures of the chick brain: effects of exogenous melatonin. Brain Res Bull 57:603-11.

Cassone VM, Menaker M (1984) Is the Avian Circadian System a Neuroendocrine Loop? J Exp Zool 232:539-549.

Cassone VM, Moore RY (1987) Retinohypothalamic projection and suprachiasmatic nucleus of the house sparrow, Passer domesticus. J Comp Neurol 266:171-82.

Cassone VM, Speh JC, Card JP, Moore RY (1988) Comparative anatomy of the mammalian hypothalamic suprachiasmatic nucleus. J Biol Rhythms. 3:71-91.

Cassone VM, Brooks DS, Hodges DB, Kelm TA, Lu J, Warren WS (1992) Integration of circadian and visual function in mammals and birds: brain imaging and the role of melatonin in biological clock regulation. In: *Advances in Metabolic Mapping Techniques for Brain Imagins of Behavioral and Learning Functions*. Kluwer Academic Publishers, Dordrecht/Boston/London: 299-318.

Cassone VM, Lu, J (1992) The pineal gland and avian circadian organization: The Neuroendocrine Loop. Adv Pineal Res 8:31-40.

Ebihara S, Uchiyama K, Oshima I (1984) Circadian organization in the pigeon, *Columba livia*: the role of the pineal organ and the eye. J Comp Physio. A 154: 59-69.

Gaston S, Menaker M (1968) Pineal Function: The Biological Clock in the Sparrow? Science 160:1125-1127.

Klein DC, Moore RY and Reppert SM (1991) Suprachiasmatic Nucleus: The Mind's Clock. Oxford: Oxford University Press.

Lu J, Cassone VM (1993) Daily melatonin administration synchronizes circadian patterns of brain metabolism and behavior in pinealectomized house sparrows, Passer domesticus. J Comp Physio A 174:775-82.

Rivkees SA, Cassone VM, Weaver DR, Reppert SM (1989) Melatonin receptors in chick brain: characterization and localization. Endocrinology 125:363-368.

Yasuo S, Yoshimura T, Bartell PA, Iigo M, Makino E, Okabayashi N, Ebihara S (2002) Effect of Melatonin administration on qPer2, qPer3, and qClock gene expression in the suprachiasmatic nucleus of Japanese quail. Eur J Neurosci 16:1541-1546.

Zimmerman NH, Menaker, M (1979) The pineal gland: A pacemaker within the circadian system of the house sparrow. Proc Natl Acad Sci 76:999-1003.

CONTACT INFORMATION

Name:	Amanda Clauson
Address:	Undergraduate Department of Biology, Texas A&M University, College Station, TX 77843-3258
Email address:	amandaclauson@gmail.com
Education:	Graduated from Langham Creek High School May 2003. Expected to graduate suma cum laude from Texas A&M University with a B.S. in cell and molecular biology in May 2007.